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Microglia and the Aging Brain: Are Senescent Microglia the Key to Neurodegeneration?

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ABSTRACT

The single largest risk factor for aetiology of neurodegenerative diseases like Alzheimer's disease is increased age. Therefore understanding the changes that occur as a result of aging is central to any possible prevention or cure for such conditions. Microglia, the resident brain glial population most associated with both protection of neurons in health and their destruction is disease could be a significant player in age related changes. Microglia can adopt an aberrant phenotype sometimes referred to either as dystrophic or senescent. While aged microglia have been frequently identified in neurodegenerative diseases such as Alzheimer's disease, there is no conclusive evidence that proves a causal role. This has been hampered by a lack of models of aged microglia. We have recently generated a model of senescent microglia based on the observation that all dystrophic microglia show iron overload. Iron-overloading cultured microglia causes them to take on a senescent phenotype and can causes changes in models of neurodegeneration similar to those observed in patients. This review considers how this model could be used to determine the role of senescent microglia in neurodegenerative diseases.

Abbreviations:

AD: Alzheimer's disease

CNS: central nervous system

CSF1: colony stimulating factor 1

CSFR1: CSF1 receptor

Eif2a: Eukaryotic translation initiation factor 2A

HDLS: hereditary diffuse leukoencephalopathy with spheroids

IFNγ: interferon gamma

IL: interleukin

Kv1.3: Potassium voltage-gated channel 3

LPS: Lipopolysaccharide

LRRK2: Leucine rich repeat kinase 2

mTOR: mammalian target of rapamycin

PD: Parkinson's disease

ROS: Reactive oxygen species

SA-β-GAL: Senescence-associated beta-galactosidase

SASP: senescence associated secretory phenotype

SH-SY5Y: a human neuroblastoma cell line

SIRT-1: sirtuin 1

TGFβ: Transforming growth factor beta

TLR: Toll-like receptor

TNFα: Tumor necrosis factor alpha

TREM2: Triggering receptor expressed on myeloid cells 2

sTREM2: soluble TREM2

INTRODUCTION

Human aging results in gradual loss of normal functioning. Changes at the cellular level are believed to contribute to this process (Lopez-Otin *et al.* 2013). Aging within the human brain is of considerable interest because age related changes there are frequently irreversible and can lead to changes that severely impair normal activity (Damoiseaux 2017). Furthermore, the changing cellular environment of the aging brain is the single largest contributing factor to the increased likelihood of developing a neurodegenerative disease such as Alzheimer's disease (AD) (Pierce *et al.* 2017).

Our understanding of the cause of aging is limited. Our population currently experiences unprecedented long-life expectancy, which implies that the incidence of age-related diseases, like neurodegenerative disease, will increase dramatically. Therefore, there is a great need to more fully understand the aging process in order to circumvent those aspects which initiate neurodegenerative diseases. Understanding aging at a systemic level is equally challenging, implying the best current approach is to understand cellular aging (Barzilai *et al.* 2018). For the brain, this implies understanding how the molecular processes in neurons and glial cells change as we age and also how the interactions of these cells are affected by such changes.

Neuronal survival and normal function may be affected by cellular changes that are internal to them. However, neurons are highly dependent on glia for many reasons (York *et al.* 2018). Therefore, the aging of glia can have significant impact on neuronal viability as well as the proteins they express or even protein turn-over (Verkhratsky *et al.* 2014, Brown 2009). The potential role of glia in neurodegenerative disease has been discussed for some decades now (Henstridge

et al. 2019, Itagaki *et al.* 1989). However, such studies have largely focussed on abnormal glia behaviour such as activation of microglia or the formation of astrocyte scars and the secretion of pro-inflammatory cytokines (Liddelow 2019). However, there is increasing evidence that glia cells undergo an aging process and this may also impact neurodegenerative disease progression (Holtzman & Ulrich 2019, Cohen & Torres 2019, Spittau 2017). These glial cells can be described as either senescent or dystrophic (Streit *et al.* 2014). Similar in some ways to the activated state, such glia secrete an altered array of molecules, especially cytokines and also lose supportive roles that normally shield neurons from various assaults (Lopes *et al.* 2008).

It is currently unclear whether the two terms (senescent and dystrophic) describe one state or are actually two different states. Senescent can also imply different states. Originally derived from the study of cancer cells, cellular senescence implied a loss of the ability to divide (Campisi 2013). However, more recently this has come to imply an age related change in the secretory profile, as in the senescence associated secretory phenotype (SASP) (Coppe *et al.* 2008, Chinta *et al.* 2015). Dystrophic on the other hand is a term derived from a morphological change as observed from the study of brain sections (Streit *et al.* 2004). These changes in cell structure cannot be easily associated with a molecular change in cells as there is currently no easy way to identify such cells outside of these morphological differences (Streit et al. 2014). A recent publication has highlighted how critically important understanding glial aging may be. The promoter for a molecule associated with cell cycle arrest ($p16^{Ink4a}$) was used to drive the expression of a suicide protein in glial cells in a transgenic mouse model (Bussian *et al.* 2018). In this tau based model of neurodegeneration, the destruction of senescent (non-dividing) glia cells

greatly reduced observed pathology. This implies that destruction of senescent glia may greatly benefit prognosis in cases of neurodegenerative diseases. However, what remains unclear is how this would be achieved in patients or exactly which population of glia should be targeted.

Impairment of neuronal activity seen in both aging and diseased states is mostly likely to be caused by microglia that have become senescent/dystrophic. However, a conclusive demonstration of a role for senescent microglia in neurodegenerative disease remains absent. Such evidence requires the ability to specifically identify and target these cells. To this end there have been recent attempts to create *in vitro* models of senescent microglia and verify that they induce changes in neurons that reflect changes seen in neurodegenerative diseases (Angelova & Brown 2018b, Angelova & Brown 2018a). This review will present the evidence that such new models of senescent microglia are paving the way to their *in vivo* identification and a fuller elucidation of their role in brain aging and disease.

ROLE OF MICROGLIA

Neurons and macroglia such astrocytes and oligodendrocytes share a similar embryonic origin, deriving from the neural tube which arises from the ectoderm (Stiles & Jernigan 2010). Microglia in comparison, have an origin outside of this and enter the brain following migration and take up residence there prior to the formation of the blood brain barrier. The exact origin of microglia has been subject of some controversy. While it is well established that microglia derive from macrophages that migrate into the developing nervous system, the initial starting place for that migration has different potential sources. Initial experiments in mice suggested that

microglia had a bone marrow origin (Hickey & Kimura 1988). It was later suggested that the radiation used to generate mouse chimeras caused brain inflammation which was the reason for the migration of bone marrow precursors (Mildner *et al.* 2007). It is now accepted that microglia are derived from yolk-sac macrophage precursors and are genetically different from blood mononuclear cells (Prinz & Priller 2014).

Functionally, microglia are largely considered to be the immune cell of the brain, remain in non active or surveillance state (ramified) unless induced to activate to deal potential threat to the neurons (Figure 1). Microglia play an important role in development of the CNS and also CNS homeostasis in health and disease. They are not the only immune cell in the brain. Other immune cell types include but are not limited to macrophages and lymphocytes (Strazielle *et al.* 2016).Perivascular, meningeal and choroid plexus macrophages also reside at the CNS interface (Goldmann *et al.* 2016). Additionally, peripheral macrophages are capable of migrating through the blood-brain barrier. However, unlike other macrophages, microglia haven't been found to be continuously renewed from myeloid progenitor blood cells and instead they increase in number through cell division (Askew *et al.* 2017). As the microglial population is predefined early in development it is not surprising that they are vulnerable to physiological disturbances such as ageing that can contribute to the development of psychiatric and neurodegenerative diseases.

Until recently, microglia were viewed under the misconception that they were static bystanders and only acted in conditions of injury or disease. Now it has been shown that microglia constantly interact with different CNS components and have a central role in the maintenance of brain homeostasis. In development microglia migrate from

the yolk sac to the CNS at approximately the same time neurons are formed. Thus, microglia participate in many important events in the developing CNS such as neurogenesis, apoptosis, synaptic pruning and modelling of neural networks (Pont-Lezica *et al.* 2014, Hoshiko *et al.* 2012, Paolicelli *et al.* 2011, Prinz & Priller 2014).

In the adult organism microglia maintain that multifunctionality and are capable of quickly adapting to changing conditions to adopt a variety of states. In the healthy brain microglia maintain a quiescent “surveillance” phenotype which is maintained by soluble factors secreted by healthy neurons and increased levels of microRNA-124 (Conrad & Dittel 2011). Microglia in the surveillance state (previously known as the resting state) can be recognized by their ramified morphology. Ramified microglia constantly screen the brain with their highly motile processes for signs of damage-associated molecular pattern molecules (DAMPs) such as ATP or calcium release (Nimmerjahn *et al.* 2005). The most morphologically adaptable and motile cell in the brain, they have been reported to constantly extend and protract their processes and be able to contact the synapses of neurons and secrete communication molecules to regulate them (Panatier & Robitaille 2012, Hristovska & Pascual 2015).

If signs of damage are detected the microglia migrate to the site of injury and convert to an activated or reactive state (Nimmerjahn et al. 2005). The morphology of microglia is an identifying characteristic of their activation state. Non-phagocytic reactive microglia display a thickening of their branches, upregulation of MHCI/II, secretion of proinflammatory cytokines and reactive oxygen species (ROS) (Colton 2009, Rock *et al.* 2004, Gehrmann *et al.* 1995). As they progress in their activation they can assume a phagocytic state which is characterised by a large ameboid shape and in addition to the aforementioned inflammatory signals they gain the

ability to phagocytose material and display it for T-cells (Gehrmann et al. 1995, Rock et al. 2004, Aloisi 2001). There is also evidence that microglia can support re-myelination following axonal damage. Depletion of microglia in experimental models of re-myelination showed significant impairment implying a supportive role for microglia (Lloyd & Miron 2019).

It is thought that depending on the signals they receive microglia can polarise to states similar to M1 and M2 in macrophages where M1 is pro-inflammatory and M2 is phagocytic and aids in tissue regeneration (Ransohoff 2016). However, the M1/M2 model fails to account for the complexity of the brain environment and the variety of signals microglia are exposed to by different cell types. Sequencing of microglial transcriptomes in surveillance and reactive states has found their reactive signature is diverse and difficult to characterise (Hirbec *et al.* 2018, Wes *et al.* 2016). Some have argued that the M1/M2 distinction should be done away with altogether as it was developed through *in vitro* experiments in a simplified environment that cannot be replicated *in vivo* (Ransohoff 2016). In the human brain microglia are found to adopt intermediate activated phenotypes demonstrating the complex role these myeloid cells are expected to play in the brain. That has led to the agreement that describing activation states in macrophages including microglia is best done by referring to the stimulating molecule that produced the reactive state (Wes et al. 2016). Just as it has been proven difficult to distinguish specific activation states in microglia on the molecular level, so it has been hard to identify the specific morphology of those cells in live tissue. Historically, most observations of microglia have focused on diseased states and on fixed tissue. As mentioned above microglia are highly reactive cells that can change both their morphology and their molecular signature with changing conditions. With the emergence of more accurate

techniques to image live tissue such as two-photon laser scanning microscopy we are beginning to get a better understanding of microglial motility and morphological spectrum in physiological conditions (Hristovska & Pascual 2015, Stopper *et al.* 2018).

Microglial priming is a process driven by changes in the molecular environment including exposure to molecules that drive proliferation (Perry & Holmes 2014). It consists of the increased reactivity of microglial cells upon stimulation. It has been reported in aged mice upon stimulation with IL-1β and IL-12 (Lee *et al.* 2013). Two types of priming have been reported, classic which has been linked to exposure to IFN-γ and is neurotoxic, and alternative – linked to exposure to IL-4 and IL-13 and suggested to be neuroprotective (Hickman *et al.* 2013). Toll-like receptors 2,3 and 4 have been shown to be essential for the classical priming process (Facci *et al.* 2014). The transcriptional signature of microglial priming seems to be dependent on the High mobility group box 1 (HMGB1) and inhibiting it prevents microglia from entering a primed state (Holtman *et al.* 2015, Fonken *et al.* 2016).

DYSTROPHIC AND SENESCENT MICROGLIA

Evidence of microglial aging was first identified in the brains of aged individuals by morphology and immunohistochemistry (Streit et al. 2004). They were found to be widespread and were characterised by a dystrophic morphology that included process deramification, shortening, gnarling and beading, the formation of spheroids and cytoplasmic fragmentation (Figure 2). It was this type of dystrophic microglia that were also found to store iron through elevated levels of ferritin (Lopes et al. 2008). The accumulation of iron in microglia with age is an interesting phenomenon as

microglia are not the main iron-storing cell type in the brain and iron may play a major role in age-related microglial dystrophy.

Aging microglia have also been found to have an altered surveillance phenotype with less dendritic branching and reduced process motility. When confronted with injury they exhibit lower migration rates and have a more sustained inflammatory response in reaction to damage (Damani *et al.* 2011). This characteristic increase in low-grade inflammation is typical of the aging brain with higher levels of pro-inflammatory cytokines and lower levels of anti-inflammatory cytokines detected in aged brains (Sparkman & Johnson 2008). Microglia have been found to be very long-lived cells in a study that monitored them over the mouse lifespan (Weinberg 2008). This puts microglia forward as ideal candidates for chronic senescence and explains how microglial senescence can potentially have a major effect in neurodegenerative diseases. As microglia are the main immune cells in the brain the way they affect inflammation in the brain has been studied extensively. Aged microglia have been found to not only change their cytokine signature to a pro-inflammatory one but also exhibit reduced phagocytosis and increased ROS production(Koellhoffer *et al.* 2017). These changes result in microglia not only failing to maintain neuronal health but also impairing it thus contributing to the possible development of neurodegenerative diseases. The senescence associated secretory phenotype (SASP) is a newer method of characterising aged microglia (Streit et al. 2014). SASP is an integral part of the characteristics of a senescent cell. It includes many signalling molecules such as proinflammatory cytokines, chemokines, ROS, growth factors and proteases that can cause a significant negative impact on surrounding cells and is linked to aging and neurodegenerative disease (Chinta et al. 2015).

The criteria for SASP differ from cell type to cell type, but microglia have been found to undergo SASP-congruent increases in TNFα, IL1β, IL-6 and IL-8 (Sierra *et al.* 2007). Interestingly, one of the criteria for developing SASP in a cell type is DNA damage (Coppe *et al.* 2010). DNA damage has been found to increase in aged microglia, particularly in mitochondria (von Bernhardi *et al.* 2015, Hayashi *et al.* 2008). Telomere shortening is also found in human and rat aged microglia, especially in association with dystrophy (Flanary & Streit 2004). It is likely that a SASP signature will be established for aging microglia in the near future.

The genomic signature of aged microglia has been of great interest since the advent of next generation sequencing technologies. Unfortunately attempts to characterise them have yielded varied and often conflicting results with no clear and consistent specific genetic or protein markers for aged microglia emerging (Crotti & Ransohoff 2016, Spittau 2017, Wes et al. 2016, Olah *et al.* 2018). The aged microglia in the mouse retina exhibited significant changes in genes controlling inflammation including the NF-κB signalling pathway and upregulated complement genes C3 and complement factor B (Ma *et al.* 2013). A study on the microglial sensome of aged mice reported a shift in microglia to a more neuroprotective phenotype with age with a particular decrease in genes related to sensing endogenous ligands but not phagocytosis or exogenous sensing (Hickman et al. 2013). Those two studies suggested that microglial aging is associated with neuroprotection. In contrast, another study on aged mouse microglia gene expression reported decreased expression in cytoskeletal reorganization proteins suggesting aged microglia are less motile. They also observed increased expression of cytokine genes suggesting that microglia have a reduced ability to migrate to sites of injury and stimulate inflammation, suggesting they play a less neuroprotective role (Orre *et al.* 2014). A

recent gene expression meta-analysis reported that aged microglia display a different expression signature from LPS or IL-4 stimulated microglia, suggesting they don't exhibit a classic activation state but do overlap with primed microglia which are also neurotoxic (Holtman et al. 2015). The few datasets currently published on human microglia aging transcriptome point in the direction that mouse and human microglia may age differently (Galatro *et al.* 2017, Olah et al. 2018). Olah et. al. (2018) detected significant changes in more than 2000 genes in aged microglia. The pathways that were upregulated were involved in amyloid fibril formation while the TGF-β signalling pathway was downregulated thus suggesting a more pro-inflammatory phenotype for aged microglia. Other upregulated genes were linked to SASP, cytokine signalling, DNA methylation and maintenance among others. That study also validated their expression data with proteomic data as well, finding that many proteins from the amyloid fibril formation gene set were still upregulated in aged microglia. The changes detected in the Galatro et al. (2017) study involved reduced expression of genes related to the actin cytoskeleton and cell surface sensor receptors and alterations in immune response genes. Comparison of this dataset with an existing mouse dataset found very limited overlap in gene expression changes between the two organisms. It is possible that this is due to isolation methods of the cells that may alter their gene expression, but it could also be due to microglia exhibiting different aging signatures in different parts of the brain (Grabert *et al.* 2016). The differences detected between human and mouse datasets could potentially be explained by the vast disparity in the lifespans of the organisms and of the microglia themselves as a consequence but also by the environments that humans are exposed to that laboratory mice never would. It seems that gene expression studies can reproducibly detect changes in larger networks in aged

microglia such as inflammatory response, cytoskeletal remodelling, and ligand sensing; but it's difficult to identify specific biomarkers for identifying aged microglia just from expression data (Holtman et al. 2015).

The description of "aged" microglia may include a number of different distinct phenotypes and currently the term is loose and uncertain (Koellhoffer et al. 2017). As mentioned above, the term dystrophic describes all the visually altered microglia in the aged brain, identifiable by their elevated iron content (Simmons *et al.* 2007). However, "senescent" microglia may include these cells and others. Indeed, the term senescent may not be sufficient to cover all "aged" microglia and there may be a fine line between such microglia and primed microglia which may simply be more abundant with age (Niraula *et al.* 2017). Furthermore, it has recently been suggested that if senescence is strictly defined as cell that can no longer divide, then a second "aged" phenotype call be described which has been termed epigenetic aging (Kabacik *et al.* 2018). This suggestion comes from observations on methylation patterns that change with age and may contribute to altered gene expression patterns that result from the aging process. However, whether this is important for microglia remains to be determined and presently the SASP remains the only widely accepted phenotype for aged microglia.

Cellular senescence has been shown to occur in many replication competent cell types in the brain, as well as microglia, and is thought to be a major contributor to inflammation in the brain in old age (Chinta et al. 2015). As mentioned above senescence is classically characterised by arrested growth due to elevated DNA damage and oxidative stress that results in increased SA-β-GAL activity, $p16^{INK4a}$ expression and the SASP among others. In microglia, iron accumulation seems to be the single clearest way to identify senescent microglia, while whether they can still

proliferate or not cannot be assessed. Interestingly, cells *in vitro* have been found to also naturally accumulate iron as a characteristic of the process of acquiring senescence (Masaldan *et al.* 2018, Killilea *et al.* 2003).

A truer understanding of aged/senescent/dystrophic microglia will only come about with a valid way identifying them. Once a valid molecular tag can be found this will lead to their isolation and characterisation. In chronic senescence which would be the type present in brain microglia, SASP can be more heterogenous than in cases of *in vitro* replicative senescence (van Deursen 2014). Nevertheless, understanding better how microglia undergo senescence by characterising their specific senescent phenotype and SASP is very important for determining how they affect the molecular environment of the aging brain, especially in terms of inflammation.

ROLES OF MICROGLIA IN NEURODEGENERATION

A role for microglia in neurodegenerative disease has been postulated for some time (Hickman *et al.* 2018, McGeer *et al.* 1993). This is because activated microglia have been found in association with disease markers such as plaques of β-amyloid in AD (Mattiace *et al.* 1990). However, there has been recent discussion as to whether these microglia are activated or dystrophic and which form is mostly likely to be causative in the relevant pathological changes (Navarro *et al.* 2018). Even considering a possible activated state, evidence suggest that both markers of M1 and M2 activation states are present in AD (Hopperton *et al.* 2018). Other studies suggest both activation states are present at different times (Tang & Le 2016). Single-cell RNA-sequencing studies also suggest that there is continuous, heterogeneous spectrum of states present in the brains of mouse models of AD (von

Maydell & Jorfi 2019). A comparison of microglial markers between individuals with AD and high-pathology but cognitively normal controls showed that microglial activation is increased in AD, suggesting that microglial activation is not simply a reaction to AD pathology such as β-amyloid deposition (Hopperton et al. 2018). It is possible microglia are pushed towards a more proinflammatory phenotype through age-related changes.

Mutations in genes related to microglial function have been linked to neurodegenerative disease risk. For example, a rare variant of the TREM2 gene leads to an increase in the risk for developing AD 3-4 times and more severe pathology. Recent evidence also suggests that TREM-2 knockout increases Aβ-related pathology (Griciuc *et al.* 2019). In the context of both aging and neurodegeneration TREM2 is thought to interact with APOE to activate pathways linked to microglial activation, survival and phagocytosis (Krasemann *et al.* 2017). Loss of function mutations in TREM2 also cause a rare deadly disease (Nasu-Hakola Disease) that presents with neurodegenerative symptoms and bone cysts (Yeh *et al.* 2017). The expression of soluble TREM2 by aged microglia of a mouse AD model was also found to rise with both amyloid load and markers of microglial activation over time, suggesting that sTREM2 also plays a role in the neurodegenerative process (Brendel *et al.* 2017). The expression of TREM-2 also impacts other neurodegenerative diseases. As an example, knockout of TREM-2 causes α-synuclein–induced neuronal loss in a model of PD (Guo *et al.* 2019).

Leucine rich repeat kinase 2 (LRRK2) is a protein expressed highly in microglia that is implicated in both microglial activation and lysosomal degradation. Mutations in LRRK2 are the most common in both familial and sporadic Parkinson’s disease

(PD). They are thought to result in increased proinflammatory signalling and possibly prevent microglial cells from degrading protein aggregates (Schapansky *et al.* 2015, Gillardon *et al.* 2012). CSF1R is a cell surface receptor for the cytokine CSF1 which in the brain is expressed predominantly in microglia and is a mediator for microglial proliferation and differentiation. CSF1R loss of function mutations have been shown to cause hereditary diffuse leukoencephalopathy with spheroids (HDLS), a neurodegenerative disease that has symptoms of dementia and parkinsonism among others (Rademakers *et al.* 2011). CD33 is another microglial cell surface receptor that has both been found to be upregulated in AD and has a rare variant that confers increased risk of developing AD. CD33 acts to reduce microglial proliferation and phagocytosis and thus exacerbates AD pathology (Griciuc *et al.* 2013, Malik *et al.* 2013). These examples show that microglial function is essential to maintaining neuronal homeostasis and health and thus any disruption in microglial function can result in neurodegeneration.

As immune cells, microglia have been found to react to the presence of misfolded proteins in both AD and PD. Microglia are well known to be activated by the presence of β-amyloid and to cluster around sites of amyloid plaques (Hansen *et al.* 2018). Exposure to β-amyloid causes a change within the metabolism of microglia changing their energy dependence to glycolysis (Baik *et al.* 2019). This impairs microglia functioning in the activated state. This could be reversed with interferon-γ, which was found to be beneficial in AD model mice. This suggests metabolic correction of microglia could be effective in the treatment of AD (Baik et al. 2019). As the main protein that aggregates in PD, α-synuclein can also interact with microglia. Microglia have been shown to react to aggregated α-synuclein but not to monomeric through TLR receptor activation (Beraud *et al.* 2013, Fellner *et al.* 2013).

The TLR2 receptor has been shown to be activated by the most likely toxic form of α-synuclein aggregates – β-rich oligomers (Kim *et al.* 2013). Additionally, microglia can also become activated through the detection of neurons under stress. Activated microglia secrete IL1β and TNFα which induce the synthesis of chemokines that have been found to contribute to neuronal demise through the recruitment of neutrophils and monocytes from blood (Rock et al. 2004). Through the secretion of proinflammatory signals and ROS activated microglia can lead to the retrograde apoptosis of neurons that are connected to the degenerating neurons thus spreading the pathology. Therefore, through inappropriate action microglia are capable of perpetuating neurodegeneration in a feedback loop of inflammatory signalling (Perry *et al.* 2010).

As phagocytes, one of the roles of microglia is to clear any cellular debris and protein aggregates that they encounter. Therefore, one of the functions of microglia is degradation of β-amyloid (Ries & Sastre 2016). However, aging microglia lose their ability to clear β-amyloid effectively from the extracellular space. In mouse models aged microglia have been found to have reduced expression of β-amyloid degrading enzymes and reduced phagocytosis (Hickman *et al.* 2008). It is thought that in AD microglial function is impaired by the mere presence of β-amyloid aggregates, thus leading to a self-perpetuating cycle of increased β-amyloid accumulation and further damage. A systematic review of microglial markers in AD found that most studies observed increases in markers related to microglial activation, but no significant difference in overall microglial markers. They also found no difference in overall cell counts suggesting that microglial number does not increase in AD, but microglial levels of activation do (Hopperton et al. 2018).

Inflammation and ineffective protein clearance are strongly linked in neurodegeneration. It has been shown that microglia play a major role in the pathology of AD and Parkinson's disease by driving inflammation in the brain (Subramaniam & Federoff 2017). In fact, in a mouse model, eliminating microglia didn't affect amyloid deposition but prevented neuronal loss and degeneration and also resulted in improved cognition (Spangenberg *et al.* 2016, Dagher *et al.* 2015). Reducing inflammation by the use of NSAIDs is also linked to a lower risk of developing PD (Gagne & Power 2010). In an organotypic cell culture model of β-amyloid accumulation in the aged brain, old microglia's ability to clear β-amyloid was rescued by the presence or the conditioned medium of young microglia, suggesting that the effects of microglial aging on amyloid deposition can be reversed (Daria *et al.* 2017).

SENESCENT MICROLIA AS THE LINK BETWEEN AGING AND NEURODGENERATION

Senescent microglia present both the impaired neuroprotective ability and the low but sustained secretion of molecules that drive inflammation seen in neurodegeneration (Koellhoffer et al. 2017). Dystrophic microglia have been identified in both aged brains and the brains of patients with neurodegenerative disease. They have been found near sites of tau pathology and amyloid plaques of AD brains and near Lewy bodies in dementia with Lewy bodies brains (Lopes et al. 2008, Streit & Xue 2014, Streit & Xue 2016, Streit *et al.* 2009, Streit 2004).

Aged microglia in models of β-amyloid load that exhibited a lower phagocytic ability were also found to express the cytokines TNFα and IL-1β, unlike microglia that did phagocytose β-amyloid (Hickman et al. 2008). The expression of inflammatory cytokines is thought to also drive further microglial damage and perpetuate the AD

pathology. Microglia have also been shown to be able to internalise and degrade extracellular α-synuclein aggregates in a cell culture study, an activity that is apparently controlled by the activation state of the cells as it was reduced by activation with lipopolysaccharide (LPS) (Lee *et al.* 2008).

The role of iron-rich microglia in neurodegenerative disease cannot be ignored. Ferritin-positive dystrophic microglia have also been found associated with amyloid plaques and neurofibrillary tangles (Streit et al. 2014). Iron also accumulates in AD brains in the hippocampus and in particular in the amyloid plaques of AD patients (Raven *et al.* 2013, Smith *et al.* 1997). It is possible that iron together with other metal ions plays a role in the toxicity of β-amyloid oligomers (Tabner *et al.* 2010). Iron appears to also accumulate excessively in regions affected by Parkinson's disease such as the *substantia nigra* and is associated with Lewy bodies where dystrophic ferritin positive microglia have been identified (Xu *et al.* 2017). Disruption in iron homeostasis has also been linked with a higher secretion of proinflammatory cytokines in microglia *in vitro* (Wang *et al.* 2013). Furthermore, short-term iron exposure has been found to induce rat primary microglia to potentiate neurotoxicity (Zhang *et al.* 2014). Exposure to iron has been linked to an increased risk of developing PD (Hare *et al.* 2017, Caudle *et al.* 2012, Peng *et al.* 2007). Neuromelanin, a protein that stores iron in neurons can be phagocytosed by microglia attracted to degenerating neurons, thus increasing the iron load of those cells. Interestingly, it has been demonstrated that neuromelanin phagocytosis can induce increased release of proinflammatory cytokines and ROS, thus driving inflammatory processes that can contribute to neuronal degeneration further (Rathnasamy *et al.* 2013). It is possible that iron accumulation in microglia could be a protective mechanism from iron toxicity in the brain which then can proceed to

damage the microglia themselves and induce the accelerated aging signature seen in neurodegeneration (Krabbe *et al.* 2013).

MODELS OF AGED MICROGLIA

The study of aging microglia is important not only in terms of understanding their phenotype and gene expression, but also in understanding what their role is in both the aging of the CNS and in neurodegenerative diseases. Thus, having models of aging microglia that can be used to study their effect on neurons is extremely important. Obviously, aging microglia can be found in aged animals and some studies have utilised them in the study of senescence (Sierra et al. 2007, Griffin *et al.* 2006, Letiembre *et al.* 2007, Stichel & Luebbert 2007, Perry *et al.* 1993, Godbout *et al.* 2005). Unfortunately, using aged animals for experiments can be exceedingly expensive and time consuming, thus making them very impractical. Additionally, despite some reported methods, isolating and maintaining aged microglia in culture has, by and large, proven to be very difficult (von Bernhardi *et al.* 2011). Some have made the case for the importance of studying age-related diseases in aged models being important enough to be worth the extra costs. However, currently it is just as difficult to obtain aged animals as ever (Johnson 2015). Transgenic models of accelerated aging are another potential avenue for observing microglial aging. The Ercc1(-/Δ) transgenic mouse model displays accelerated aging through DNA-repair deficiency (Schermer *et al.* 2013). This model has shown age-related changes in microglia (Raj *et al.* 2014). Another model of accelerated aging is the mTerc−/− mouse that exhibits telomere shortening whose microglia exhibit some signs of senescence and priming (Raj *et al.* 2015). An alternative to a full animal model can be cell culture models of aged microglia. One study has reported that microglia

isolated from neonatal mice start resembling aged microglia with time in culture, including reduced phagocytic ability, reduced motility, reduced autophagy, and changes in microRNAs and SA-β-galactosidase activity (Caldeira *et al.* 2014). However, these cells didn't exhibit the SASP expected from aging microglia. Additionally, at 16 days this model is very short lived, thus again making it impractical for the study of neurodegeneration. Another method to induce age-like changes in microglia has recently been presented by Park et al. (Park *et al.* 2019). By treating primary rat microglial cultures with the drug dexamethasone (a corticosteroid) they simulated chronic stress inflicted by steroid hormones that microglia experience over time with aging. The microglia in this model exhibited increased SA-β-galactosidase activity, increased expression of tumour suppressor genes and dysfunctional phagocytosis similarly to senescent cells. However, they also showed increased autophagy, decreased expression of inflammatory genes and decreased cytokine release which is unlike aged microglia.

The observation that dystrophic microglia can be identified by high ferritin and thus iron content provided us with a potential avenue to generate a model of aged microglia (Brown 2009). The universality of this finding suggests that this is a significant observation. Given that iron has the potential to initiate macromolecule altering oxidation through Fenton and Haber-Weiss reactions, we theorized that, even though the aged phenotype *in vivo* occurs over time, it might be possible to induce similar changes through overloading microglia with iron in culture. We used both primary mouse microglia and human and mouse microglia cell lines and exposed them to high iron concentrations. These different microglia all showed significant retention of the iron along with changes in morphology, cytokines and ROS (Angelova & Brown 2018b, Angelova & Brown 2018a). This suggests the

adoption of an altered secretory profile similar to SASP. While one of the cell lines showed a decreased rate of proliferation, this was not universally observed. However, as mentioned, it remains unclear as to whether loss of proliferative ability is a valid indicator of senescent/dystrophic phenotype for a cell type that is meant to be able to able to divide as part of its normal activities. Changes in other markers of both aging and senescence were also observed such as altered expression of the proteins SIRT-1, Kv.1.3 and altered glutamate release (Angelova & Brown 2018b, Angelova & Brown 2018a). Lastly, we looked at cellular processes that are linked to altered cellular function, namely autophagy and ER stress. By monitoring proteins associated with both processes such mTOR and Eif2a, we were able to demonstrate that our model aged microglia showed increased ER stress and decreased autophagy (Angelova & Brown 2018a). Altogether, the changes we observed in our iron-overload based model of aging microglia were similar to both senescence and dystrophic alteration (Figure 3).

While changes in the behavior and function of microglia may be indicative of them adopting an age-related phenotype, greater evidence was obtained in experiments that they are able to induce changes in a neuronal cell line that are reminiscent of changes observed in neurodegenerative diseases. One of the most respected changes in AD, associated with its onset and pathology is the accumulation of β-amyloid. We were able to induce increased survival of β-amyloid in cultures of SH-SY5Y cells when treated with conditioned medium from our model aged microglia. We demonstrated that this was the result of the altered phenotype of the microglia in that they secreted less insulin-degrading enzyme (IDE), which is one of the main enzymes associated with β-amyloid turnover. We showed that this changed in the microglia was a result of decreased autophagy-related release of IDE. This verified

that causing an aged related change in model microglia could induce an AD related change, increased levels of β-amyloid (Angelova & Brown 2018a).

In PD, the symptoms of the disease are a result of the destruction of dopaminergic neurons. The potential mechanism for this loss is death induced by toxic species of α-synuclein. The ability of α-synuclein to aggregate and generate toxic species is highly dependent on its cellular concentration. Therefore, anything that increases α-synuclein levels in cells is linked to increased risk of generating such toxic oligomers. We demonstrated that conditioned medium from our model aged microglia could induce increased expression of α-synuclein in SH-SY5Y neuronal cells through a NF-κB regulated pathway related to increased levels of TNF-α secreted by the microglia (Angelova & Brown 2018b). Additionally, we found that this conditioned medium induced increased aggregation of α-synuclein, reduced ferrireductase activity and reduced tetramer formation. All of these changes are associated with Parkinson's disease. The implication is that our model aged microglia are able to induce changes in neuronal cells related to the pathology of Parkinson's disease and adds further verification of the utility of this model to study both aging and the impact of senescent microglia on neurodegenerative disease.

We feel that given this evidence we have developed a robust and easy to generate model of aged/senescent microglia that could be used to further study both the aging process and the role of microglia in neurodegenerative diseases. However, greater support for this would be the application of this aged microglia model *in vivo*. It is this very important to determine if iron-overloaded microglia transplanted into mouse brains would induce phenotypic changes in neuronal cells similar to those seen in the culture models. For this to be achieved control and iron-overloaded microglia

could be transplanted into live rodent brains. This has been previously attempted with both primary and cell line microglia (Leovsky *et al.* 2015, Narantuya *et al.* 2010a, Narantuya *et al.* 2010b, Watanabe *et al.* 2002, Takata *et al.* 2007). The observation of either neurodegeneration or age-related changes in a transplantation model based on iron-overloaded microglia could possibly generate the ultimate aging model and reduce the time required to produce data on the nature of brain aging. However, even if this is achievable one final piece of the puzzle remains currently elusive. It is important to determine how increasing cellular iron induces a dystrophic/senescent phenotype.

MODEL MICROGLIA AND BRAIN AGING

Understanding senescent microglia and their role is not just relevant to neurodegenerative diseases. Brain aging even in non-pathological conditions is characterised by diminished cognitive ability that could be due to impaired synaptic plasticity. Maintenance of synaptic plasticity has recently been recognised as an important role for microglia both in development but also in the adult organism (Wu *et al.* 2015). Iron-fed microglia as a model for aging microglia could be used to investigate the role of microglia in these processes. A well-studied form of synaptic plasticity is long-term potentiation. Appropriate levels of ROS have been shown to play an important role in long-term potentiation in the hippocampus (Serrano & Klann 2004). Both aged and iron-fed microglia show dysregulated release of ROS which could affect these processes. Additionally, overproduction of IL-1β by microglia has also been linked to impaired long-term potentiation in the hippocampus (Patterson

2015). IL-1β levels are increased in iron-fed microglia providing another potential avenue through which brain aging could be replicated by the aged microglia model.

Genomic instability is another hallmark of aging that aged microglia could play a role in. It has been shown that chronic inflammation can lead to genomic instability in studies in the context of cancer (Lin *et al.* 2016, Yan *et al.* 2009). The fact that aged microglia drive increased inflammation in the aging nervous system suggests that they also play a role in the genomic instability seen in aging neurons (Burhans & Weinberger 2007). Lower levels of SIRT1 may be a key player in these events (Oberdoerffer *et al.* 2008) and conditioned medium from iron-fed microglia was shown to reduce SIRT1 levels in neuronal cells (unpublished data). This suggests that iron-fed microglia could potentially induce genomic instability in neurons thus expanding the role that aged microglia could play in inducing overall brain aging.

CONCLUSIONS

We began this review with the question as to whether senescent microglia could be the key to understanding the cause of neurodegenerative diseases. More than 25 years of research have pointed frequently at the role of microglia in neurodegenerative diseases. The case for this is overwhelming. The fact that microglia are also known to play a supportive positive role also clearly points to there being a specific subset of microglia being the potential culprits. Identification, of this subset has not being achieved. However, as the majority of sporadic neurodegenerative diseases occur in patients beyond the age of 50, the probability that this is subset of microglia are those with a senescent/dystrophic phenotype is extremely high. As working with aged animals is difficult then the creation of an *in*

vitro model of aging is the best possible option to increase insight into the potential role of aged microglia. Creation of a model such as ours means that aged microglia could potentially be incorporated into both *in vitro* and *in vivo* studies of these neurodegenerative diseases and also aging itself. One of the biggest potential impacts this model could have is in pioneering reversal of the effects of aging in microglia. Successfully reversing the iron-overloaded phenotype could lead to not only greater understanding of the aging process but also in powerful brain anti-aging treatments that could protect older individuals from developing debilitating neurodegenerative diseases.

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CONFLICT OF INTEREST

The authors state that there was no conflict of interest in the preparation of this review.

FIGURE LEGENDS

Figure 1 Microglia in the brain. Healthy microglial cells play a myriad of supportive roles in the brain including sensing, intercellular communication, promotion of inflammation, degradation and repair.

Figure 2 Characteristics of microglial aging. When microglia age they lose process ramification, develop process abnormalities and exhibit cytoplasmic fragmentation. They show increased iron storage and ferritin expression. Their increased release of neurotoxic substances and reduced ability to phagocytose debris and toxic protein aggregates leaves neurons vulnerable.

Figure 3 Microglial aging model induced by iron. This figure summarizes the effects caused by iron accumulation in aging microglia. It leads to an altered microglial morphology and altered intracellular processes namely increased ER stress leading to decreased autophagy. That leads to decreased release of IDE which results in decreased degradation of β-amyloid in the extracellular space. Iron accumulation also leads to increased secretion of inflammatory cytokines and ROS from which TNF-α causes increases in α-synuclein levels and toxicity in neurons mediated through FOXO3a.

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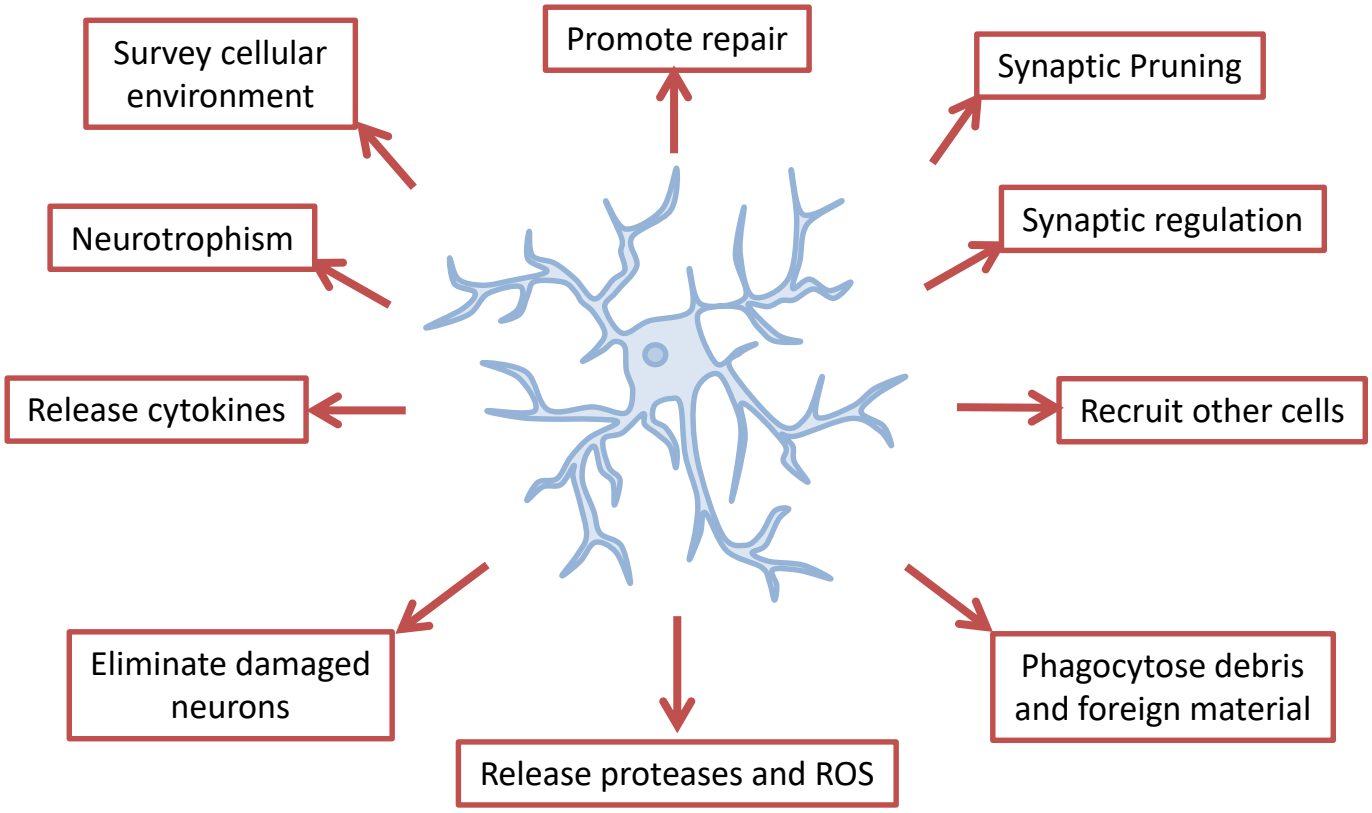
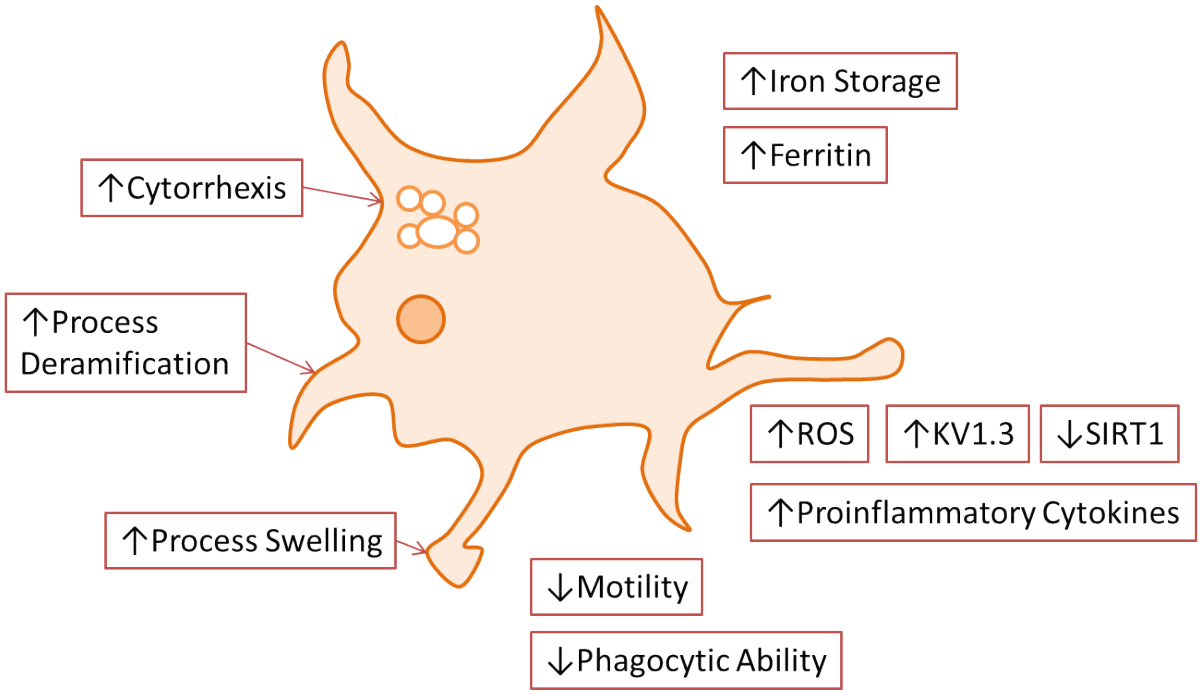
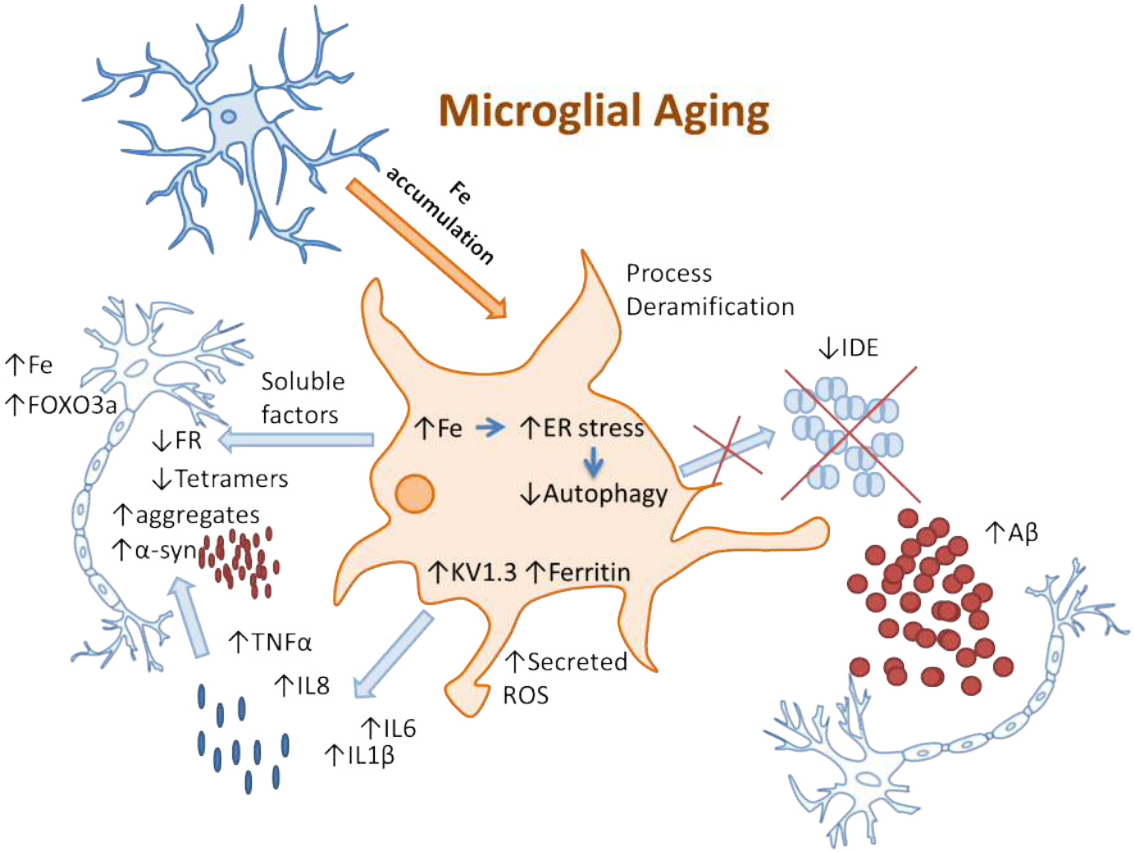
Microglia in the Brain



Microglial Senescence




Microglial Aging
Fe accumulation
Process Deramification
↑Fe
↑FOXO3a
Soluble factors
↓FR
↓Tetramers
↑aggregates
↑α-syn
↑Fe → ↑ER stress
↓Autophagy
↑KV1.3 ↑Ferritin
↓IDE
↑Aβ
↑TNFα
↑IL8
↑IL6
↑IL1β
↑Secreted ROS